J. Clin. Chem. Clin. Biochem.
Vol. 28, 1990, pp. 139–148

Myocardial Cell Damage and Breakdown of Cation Homeostasis During Conditions of Ischaemia and Reperfusion, the Oxygen Paradox, and Reduced Extracellular Calcium

By *F. Diederichs, H. Wittenberg* and *U. Sommerfeld*

Abteilung Klinische Biochemie and Abteilung Gastroenterologie und Hepatologie, Medizinische Hochschule Hannover, Hannover, FRG

(Received December 30, 1988//June 6/November 6, 1989)

Summary: Enzyme release from perfused rat heart was determined under various conditions of injury. In analogous experiments, intracellular cation concentrations were measured using ion-selective microelectrodes. Under appropriate conditions, the inhibition of mitochondrial and/or glycolytic ATP production led to a decrease in the release of enzymes. During ischaemia or the oxygen paradox, the sarcosolic Ca^{2+} concentration was highly elevated; reperfusion or reoxygenation was followed by a drastic enzyme release. This was also found to be true under the conditions of an increased permeability brought about by a reduced extracellular Ca^{2+} concentration of 0.1 mmol/l. The intracellular pH under all conditions of injury was only moderately decreased. The sarcosolic Na^+ concentration was markedly increased whereas the K^+ concentration was decreased. The critical Ca^{2+} concentration of the sarcosol beyond which cell damage and enzyme release are inducible was assumed to be in the range between 10 and 32 μmol/l. The driving force of the Na^+/Ca^{2+} exchange reaction of the sarcolemma is discussed in relation to recovery from hypoxic injury and the potential for avoiding cell damage.

Introduction

Cellular energy transductions proceed largely as a cation flow through electrochemical potential differences occurring at cell membranes. Thus, the viability of cells is intimately connected with cellular cation homeostasis and, therefore, loss of viability is associated with dissipation of these potential differences and a breakdown of cation homeostasis. The most striking change of electrochemical potential difference during cell injury can be observed for Ca^{2+}. In the case of myocardial cells, a calcium overload has been reported during the Ca^{2+} paradox (1), ischaemia and reperfusion (2), and the oxygen paradox (3). In this context, the question of whether an increase in the sarcosolic free Ca^{2+} concentration, $[Ca^{2+}]_i$, is the trigger for cell damage and enzyme release or merely a concomitant phenomenon of secondary importance is still unanswered.

Recently, we developed a hypothesis concerning the occurence of cell damage and the concomitant release of sarcosolic enzymes, which brings the membrane lesions and increased $[Ca^{2+}]_i$ into a causal relationship (4).

An additional aim of this study was to demonstrate that during ischemia and reperfusion, the oxygen paradox, and at an elevated permeability of the sarcolemma by 0.1 mmol/l $[Ca^{2+}]_e$, an increased $[Ca^{2+}]_i$ is the necessary prerequisite for the induction of cell damage. Our results of enzyme release experiments and cation-selective microelectrode measurements show that under all conditions of severe injury, a drastic release of enzymes is always preceded by a large increase of $[Ca^{2+}]_i$. The role of ATP production and swelling is discussed with respect to cell damage.

Materials and Methods

Experimental

Enzyme release and microelectrode experiments were performed with isolated, *Langendorff*-perfused rat hearts (5). During microelectrode measurements, the perfused myocardium was plunged into a thermostatted (37 °C) glass vessel, which contained the respective perfusion medium. In this way, we ensured that the whole electrochemical cell assembly was temperature equilibrated. Microelectrode impalements were carried out using arrested myocardia. Values of the respective cation concentrations were measured during a 1 – 2 min stable microelectrode impalement of one cell. During perfusion, several myocytes were successively impaled and the data for a given experiment were usually obtained from 4 hearts.

Enzymes

Perfusion media (mmol/l)

Krebs-Ringer medium: NaCl, 118; KCl, 4.7; KH_2PO_4, 1.2; $MgSO_4$, 1.2; $NaHCO_3$, 24; $CaCl_2$, 1;

Choline medium: the NaCl (118 mmol/l) of *Krebs-Ringer* medium was replaced by equimolar choline chloride;

Perfusion media were gassed with carbogen or, for hypoxic conditions, with 95% N_2 + 5% CO_2, $pO_2 \sim 2$ kPa.

Unless otherwise stated, the glucose concentration was 10 mmol/l.

Perfusion was carried out at 37 °C and at pH 7.4.

Catalytic activities were assayed using commercial kits from Boehringer Mannheim.

Microelectrodes

Cation-selective liquid membrane microelectrodes were constructed from double-barrelled borosilicate tubing (2.6 mm outside diameter) according to *Amman* et al. (6). For a full description of construction and electrical equipment see l. c. (5). The silanization procedure was improved by using a solution of dichlormethylsilan in carbon tetrachloride and tetrahydrofurane (in volume fractions: 0.035, 0.615, and 0.35, respectively). Membrane cocktails for microelectrodes were prepared according to l. c. (6). Single-barrelled microelectrodes were fabricated from borosilicate glass capillaries with filament of 1.2 mm outside diameter.

Electrode calibration

The calibration of microelectrodes was also carried out at 37 °C, since the electrochemical cell was maintained at this temperature during measurements of the perfused heart.

Ca^{2+}-selective microelectrodes were calibrated by titrating a solution containing Ca^{2+} and ethylene diaminetetraacetic acid (EDTA) and recording the pH by means of a glass macroelectrode. The free Ca^{2+} concentration was calculated from measured pH values (5). Temperature corrections for 37 °C were made with the aid of constants from l. c. (5), using *van't Hoff*'s equation.

Na^+-selective and K^+-selective microelectrodes were calibrated by varying $[Na^+]$ and $[K^+]$, respectively, but by keeping the sum of both concentrations constant.

H^+-selective microelectrodes were calibrated by means of a pH glass electrode.

Calibration solutions (mmol/l)

Ca^{2+}: Na_2 EDTA, 5; $CaCl_2$, 1; KCl, 163;

Na^+/K^+: NaCl + KCl, 163; $MgCl_2$, 1; Na_2HPO_4, 3.8; NaH_2PO_4, 1.2;

H^+: KCl, 100; NaCl, 63; $MgCl_2$, 1; $NaHCO_3$, 10; K_2HPO_4, 5; CO_2, 1.2.

For the sake of simplicity, all microelectrodes with the exception of the H^+-selective microelectrode were calibrated with reference to the concentration of the respective ion in the calibration solution. this is only permissible, however, if the activity coefficients of the calibration solution and the sarcosol do not differ greatly. Therefore, the ionic strength of the calibration solutions was adjusted to the physiological range of about 180 mmol/l. If the sarcosolic ionic strength is expected to change, e. g., by a markedly increased $[H^+]_i$, intracellular recordings should be related preferentially to ion activity of the calibration solution.

Calibration curves were constructed by polynomial approximation using the *Gauss-Jordan* algorithm. For example, a polynomial of the 5th degree was usually applied for Ca^{2+} calibration curves.

Calculations

Enzyme release from *Langendorff*-perfused isolated rat hearts at any given time was defined as the quotient of catalytic activity of perfusate over total catalytic activity of the myocardium. Swelling was expressed as the relative change in wet weight.

For the sake of clear presentation of the figures only the greatest standard error of the mean (SEM) of data is given (figs. 1, 3, 5, and 7); for microelectrode data only the span of n values is indicated.

Results

Sarcosolic cation concentrations and the membrane potential under control conditions

The shaded columns in figures 2, 4, 6, and 8 represent time-averaged control values of sarcosolic cation concentrations and the membrane potential, which were obtained from arrested hearts in separate experiments during 30 min of perfusion with *Krebs-Ringer* bicarbonate buffer + 20 µmol/l verapamil. The double-barrelled micropipettes usually had a resistance between 10 and 20 MΩ when filled with 3 mol/l KCl and measured in 0.163 mol/l KCl solution at 23 °C. To reduce leakage from the reference barrel, ion-selective microelectrodes were filled with 0.163 mol/l KCl; K^+-selective microelectrodes normally contained 0.163 mol/l NaCl. The rather positive membrane potential (−57 mV) found with these microelectrodes was further investigated by using single-barrelled micropipettes of higher resistance. With 0.163 mol/l KCl-filled electrodes (resistances 70 – 120 MΩ) we found the same value for the membrane potential (−57 ± 1 mV; mean ± SEM; n = 23). In contrast, with 3 mol/l KCl-filled single-barrelled microelectrodes (resistances 33 – 46 MΩ) a value of −73 ± 1 mV (n = 18) was found, while a double-barrelled microelectrode filled with 3 mol/l KCl gave a value of −79 ± 1 mV (n = 22). Surprisingly, with double-

barrelled K^+-selective microelectrodes, the reference barrels of which contained 3 mol/l Li acetate, revealed -72 ± 2 mV (n = 13) for the membrane potential and 0.98 ± 0.02 for pK (= 104 mmol/l), i.e. about twice the $[K^+]_i$ found with 0.163 NaCl-filled microelectrodes.

Ischaemia and reperfusion

Paradoxical cell damage by reperfusion following an extended period of ischaemia (> 60 min) and the oxygen paradox are well known phenomena of experimental heart physiology, which, however, have not been satisfactorily explained. Our hypothesis of cell damage could supply an explanation for both processes of injury, provided swelling and/or the presence of ATP coincide with an elevated $[Ca^{2+}]_i$ (see l. c. (4)).

Figure 1 shows the release of two sarcosolic enzymes of greatly different molar masses, lactate dehydrogenase and creatine kinase, during reperfusion after 90 min of total ischaemia. A 10 min long hypoxic preperfusion interval was included to avoid too strong an accumulation of acid which otherwise could have inactivated the enzyme activities as well as decreased the sarcosolic ionic strength and thereby increased the activity coefficients. Cell swelling by lactate accumulation may have been partially prevented (see discussion). Both enzymes show almost identical release kinetics. Release of the mitochondrial matrix enzyme glutamate dehydrogenase was negligible. This release behaviour was also observed during the subsequent experiments. Interestingly, enzyme release was suppressed below the hypoxic control by inhibition of the mitochondrial respiratory chain by antimycin A, despite the fact that under these special control conditions reperfusion was carried out with hypoxic *Krebs-Ringer* medium ($pO_2 \sim 2$ kPa) without glucose.

The hearts were quiescent after about 13 min (10 min of hypoxic preperfusion + 3 min of ischaemia) and appeared contracted. Similar behaviour could be observed with hearts under hypoxic conditions during the course of the oxygen paradox.

Figure 2 shows averaged negative logarithms of sarcosolic cation concentrations. A 10 min interval of hypoxic preperfusion was included. The most striking change was found for the sarcosolic pCa and, thus, for the chemical potential of sarcosolic calcium. Following a relatively short period of ischaemia (+ 10 min of hypoxia) $[Ca^{2+}]_i$ was markedly elevated above 10 µmol/l (pCa < 5). Obviously, this drastic increase of $[Ca^{2+}]_i$ had already occurred to a large extent during the ischaemic period before reperfusion was begun. Similar behaviour was observed for $[Na^+]_i$;

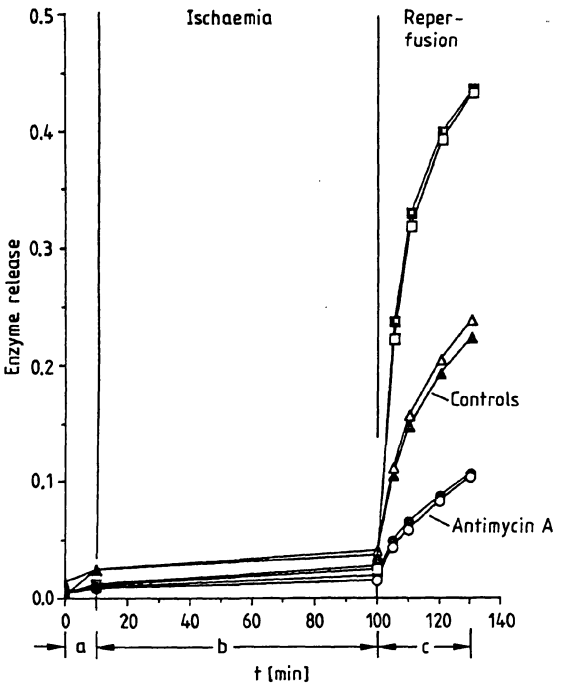

Fig. 1. Enzyme release (ratio of catalytic activity of perfusate over total catalytic activity of the myocardium) during ischaemia and reperfusion.
a) 10 min of preperfusion with hypoxic *Krebs-Ringer* medium without glucose (pO_2 2 kPa);
b) 90 min of total ischaemia; hearts were incubated in N_2/CO_2-gassed *Krebs-Ringer* medium;
c) 30 min of reperfusion with *Krebs-Ringer* medium without glucose.
Krebs-Ringer medium: SEM ≤ ± 0.04, n = 4;
Controls = hypoxic *Krebs-Ringer* medium: SEM ≤ ± 0.045, n = 4;
Antimycin A = *Krebs-Ringer* medium + 2 µmol/l antimycin A present during 130 min: SEM ≤ ± 0.013, n = 4.
Closed symbols: lactate dehydrogenase;
open symbols: creatine kinase.

the membrane potential increased continuously (became more positive) during ischaemia and did not correspond to the K^+ distribution according to the *Nernst* equation. This discrepancy was also found in subsequent measurements. $[H^+]_i$ was only moderately increased during ischaemia but was markedly affected by reperfusion.

The oxygen paradox

When hearts were perfused with oxygen-free and substrate-free *Krebs-Ringer* medium, lactate dehydrogenase and creatine kinase were not considerably released during 100 min of hypoxia (fig. 3). Reintroduction of oxygen, however, induced a rapid increase in enzyme release. This phenomenon is known as the oxygen paradox. The release kinetics during reoxygenation are very similar to those of figure 1, illustrating the similarity of both processes. Enzyme release under

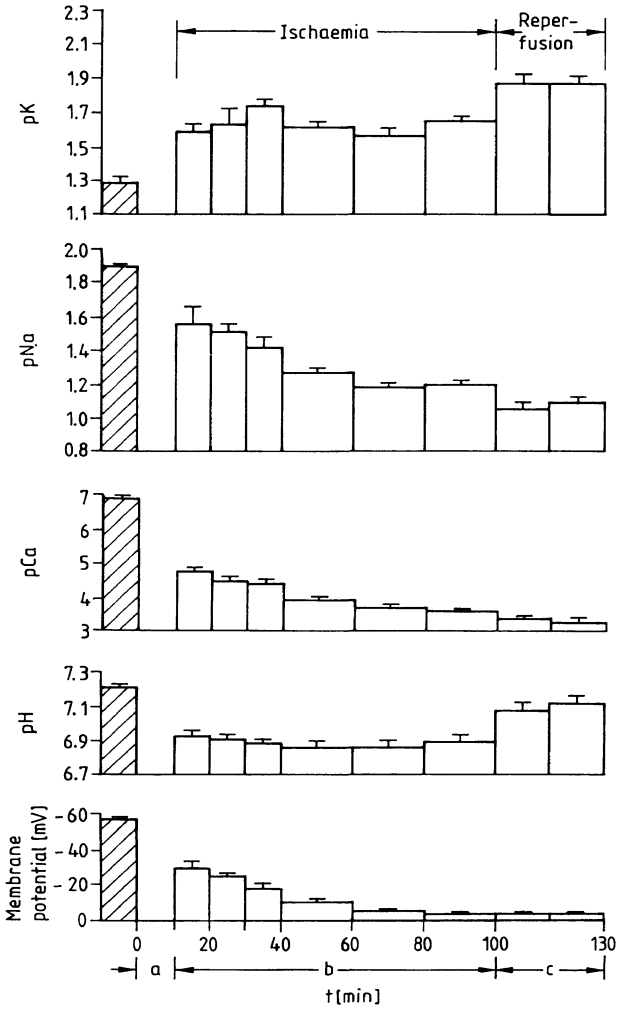

Fig. 2. Negative logarithms of sarcosolic cation concentrations and the membrane potential during ischaemia and reperfusion. Perfusion sequence a–c as in fig. 1. *Krebs-Ringer* medium without glucose. pK: n = 6 – 36; pNa: n = 5 – 34; pCa: n = 5 – 41; pH: n = 12 – 27; membrane potential: n = 28 – 132.
Hatched columns: control values of verapamil-arrested hearts.

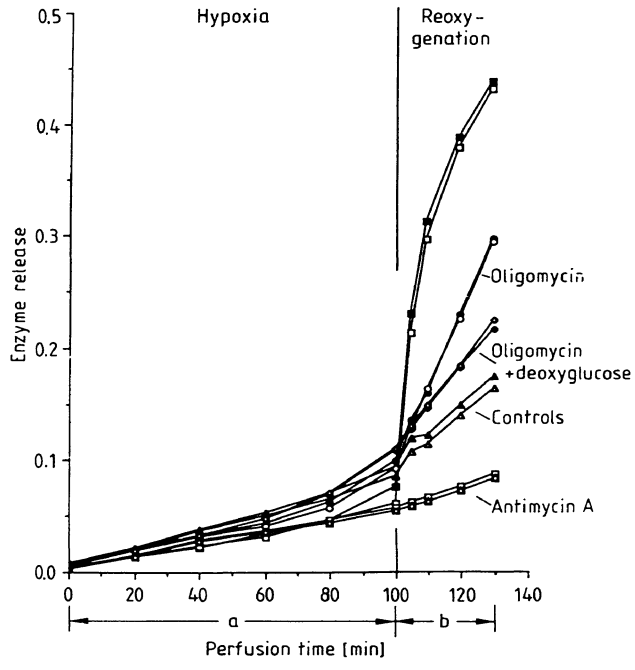

Fig. 3. Enzyme release (ratio of catalytic activity of perfusate over total catalytic activity of the myocardium) during the oxygen paradox (1 mmol/l $[Ca^{2+}]_e$).
a) 100 min of perfusion with hypoxic *Krebs-Ringer* medium without glucose;
b) 30 min of perfusion with normoxic *Krebs-Ringer* medium without glucose (control: hypoxic *Krebs-Ringer* medium).
Krebs-Ringer medium: SEM $\leq \pm$ 0.025, n = 4.
Controls = hypoxic *Krebs-Ringer* medium: SEM $\leq \pm$ 0.027, n = 4;
Antimycin A = *Krebs-Ringer* medium + 2 µmol/l antimycin A: SEM $\leq \pm$ 0.008, n = 4;
Oligomycin = *Krebs-Ringer* medium + 2 µmol/l oligomycin: SEM $\leq \pm$ 0.04, n = 4;
Oligomycin + deoxyglucose = *Krebs-Ringer* medium + 2 µmol/l oligomycin + 5 mmol/l deoxyglucose: SEM $\leq \pm$ 0.009, n = 4; all additions were present during 130 min.
Closed symbols: lactate dehydrogenase;
open symbols: creatine kinase.

hypoxic control conditions, however, is less pronounced than for the hypoxic reperfusion control and it was, therefore, possible to test two additional inhibitors of ATP production, oligomycin and deoxyglucose, in addition to antimycin A. The results unambiguously demonstrate that both with oligomycin (an inhibitor of mitochondrial ATP synthesis), and with deoxyglucose (an inhibitor of glycolysis), enzyme release during reoxygenation could be suppressed.

As seen under the conditions of ischaemia, the most drastic change was observed for the sarcosolic pCa (fig. 4). $[Na^+]_i$ increased even more rapidly than under ischaemic conditions. In contrast to its behaviour during ischemia, $[H^+]_i$ partially recovered during hypoxia. $[K^+]_i$ was further decreased during reoxygenation.

Table 1 shows the calculated driving forces, $\Delta\tilde{\mu}_{Ca}/F - 3\,\Delta\tilde{\mu}_{Na}/F$, for the Na/Ca-exchange reaction at the sarcolemma for the indicated time intervals during the hypoxic phase of the oxygen paradox. Differences of electrochemical potentials were calculated from known values of $[Ca^+]_e$ and $[Na^+]_e$ of *Krebs-Ringer* medium and the measured values of $[Ca^{2+}]_i$, $[Na^+]_i$, and the membrane potential (fig. 4). The sign was taken as positive for Ca^{2+} outward transport. The right column of table 1 shows values for a postulated $[Ca^{2+}]_i$ of 20 µmol/l (pCa = 4.7).

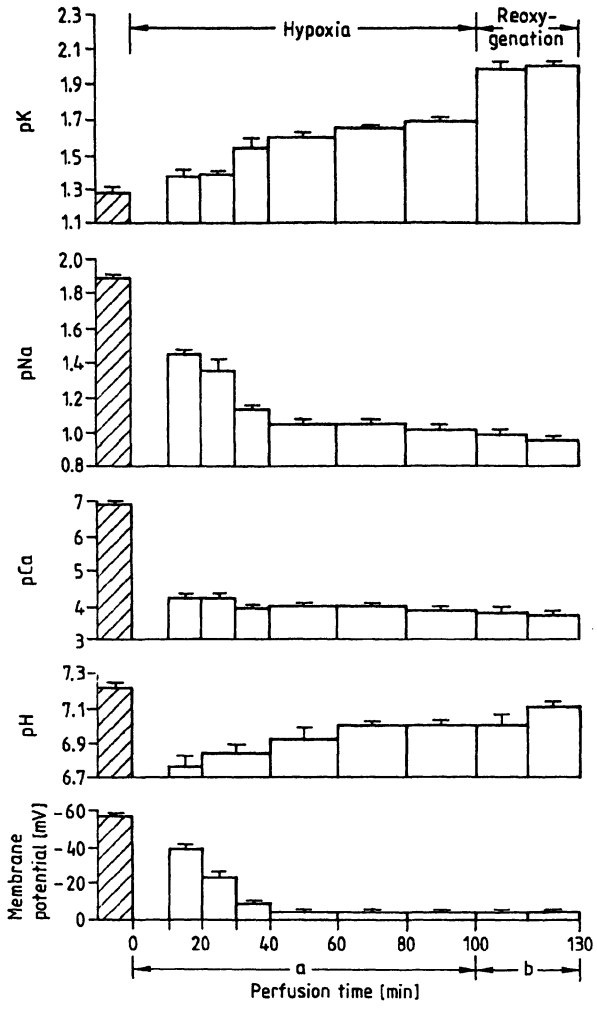

Fig. 4. Negative logarithms of sarcosolic cation concentrations and the membrane potential during the oxygen paradox. Perfusion sequence a–b as in fig. 3. *Krebs-Ringer* medium without glucose. pK: n = 3 – 38; pNa: n = 22 – 48; pCa: n = 8 – 42; pH: n = 3 – 7; membrane potential: n = 37 – 123.
Hatched columns: control values of verapamil-arrested hearts.

Tab. 1. Driving forces of the Na/Ca exchange reaction at the sarcolemma during the hypoxic phase of the oxygen paradox.
$3Na_e^+ + Ca_i^{2+} \rightleftharpoons 3\,Na_i^+ + Ca_e^{2+}$
For explanation see text.

Time interval [min]	$\frac{3\Delta\tilde{\mu}_{Na}}{F}$ [mV]	$\frac{\Delta\tilde{\mu}_{Ca}}{F}$ [mV]	Driving force $\frac{\Delta\tilde{\mu}_{Ca} - 3\Delta\tilde{\mu}_{Na}}{F}$ [mV]	
Control	−364.3	−356.5	+ 7.8	
10– 20	−231.9	−154.6	+77.3	+48.4
20– 30	−165.6	−127.0	+38.6	+12.8
30– 40	– 80.3	– 76.3	+ 4.0	−42.7
40– 60	– 49.8	– 68.8	−19.0	−63.9
60– 80	– 51.1	– 59.8	– 8.7	−62.2
80–100	– 43.6	– 56.1	−12.5	−69.7

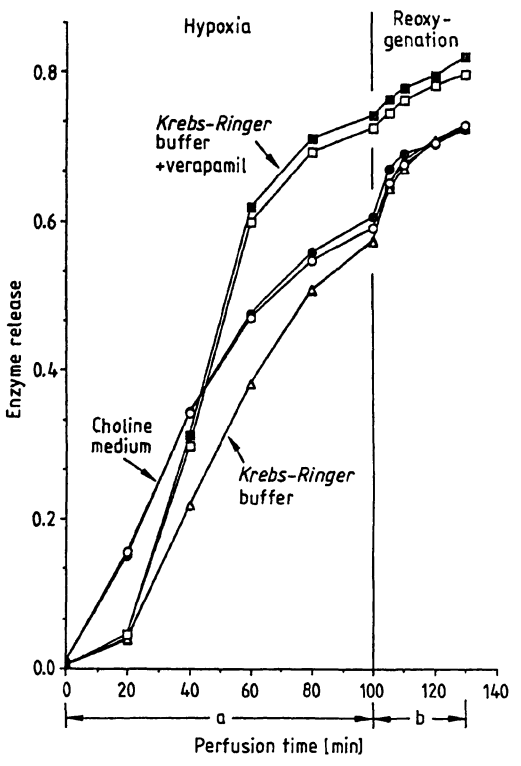

Fig. 5. Enzyme release (ratio of catalytic activity of perfusate over total catalytic activity of the myocardium) during the oxygen paradox at 0.1 mmol/l $[Ca^{2+}]_e$. Perfusion sequence a–b as in fig. 3. *Krebs-Ringer* medium without glucose: SEM ≤ ± 0.045, n = 4; choline medium (contains 118 mmol/l choline chloride instead of 118 mmol/l sodium chloride) without glucose: SEM ≤ ± 0.07, n = 4; *Krebs-Ringer* medium without glucose + 20 µmol/l verapamil: SEM ≤ ± 0.055, n = 4.
Closed symbols: lactate dehydrogenase;
open symbols: creatine kinase.

Effects of a decreased extracellular calcium concentration

Figure 5 shows the effect of a low extracellular calcium concentration ($[Ca^{2+}]_e$ = 0.1 mmol/l) on enzyme release under the conditions of the oxygen paradox. In contrast to the results of figure 3, a massive enzyme release was induced during the hypoxic perfusion interval. Moreover, a more pronounced swelling was observed, which could have been brought about only by the passive entry of ions and water and thus must have been caused by intracellular *Donnan* forces (for comparison, swelling after 130 min was 0.34 ± 0.07 and 0.57 ± 0.07 during the oxygen paradox at normal and reduced $[Ca^{2+}]_e$, respectively). A *Donnan* equilibrium between the intra- and extracellular compartment cannot be attained, however, because the cell membrane can tolerate only a relatively low hydrostatic pressure difference. Therefore, if not counteracted by active transport, the system moves towards equilibrium by increasing the cell volume. For any

given non-equilibrium distribution of permeable ions over the cell membrane, the swelling velocity depends on the permeability of the sarcolemma for the predominant ions (Na^+ and $choline^+$, respectively, and Cl^-). At normal $[Ca^{2+}]_e$ the sarolemma is almost impermeable to $choline^+$ and has a rather low Na^+-permeability, so that a *Donnan* swelling at low $[Ca^{2+}]_e$, therefore, must have been caused by increased permeability.

Figure 6 shows the negative logarithms of sarcosolic cation concentrations during perfusion under analogous conditions. $[Na^+]_i$ was more quickly elevated, but $[Ca^{2+}]_i$ was increased more slowly than under the same conditions at normal $[Ca^{2+}]_e$. $[K^+]_i$ decreased much more rapidly.

Under normoxic conditions in the presence of glucose, enzyme release at $[Ca^{2+}]_e$ of 0.1 mmol/l was inducible only with choline media. However, it could be likewise initiated by perfusion media of normal $[Na^+]_e$ when the Na/K-pump was inhibited by ouabain or withdrawal of K^+ from the perfusion media (fig. 7). Comparing the choline curves of figures 7 and 5, it is evident that under normoxic conditions, the release rate was markedly increased (2.5-fold between 20 and 40 min). Intracellular cation concentrations were measured during normoxic perfusion at 0.1 mmol/l $[Ca^{2+}]_e$ with *Krebs-Ringer* medium (+ glucose) containing ouabain (fig. 8). Again, $[Na^+]_i$ was more quickly elevated than during the hypoxic phase at normal $[Ca^{2+}]_e$. The $[Ca^{2+}]_i$ was rapidly elevated and remained fairly constant between 5 and 40 min at slightly above 10 µmol/l. The driving force of the Na/Ca-exchange reaction during 60 min was positive, i. e., Ca^{2+} transport was directed outward. Under hypoxic conditions at 0.1 mmol/l $[Ca^{2+}]_e$, this transport reaction had almost reached equilibrium after about 30 min and had became slightly negative between 40 and 50 min.

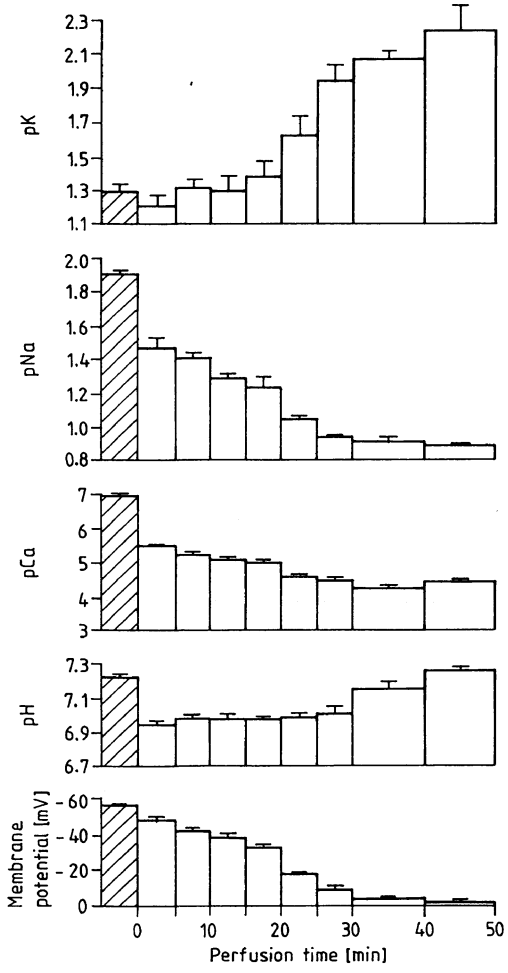

Fig. 6. Negative logarithms of sarcosolic cation concentrations and the membrane potential during hypoxia at 0.1 mmol/l $[Ca^{2+}]_e$. Hypoxic *Krebs-Ringer* medium without glucose. pK: n = 5 – 16; pNa: n = 7 – 20; pCa: n = 5 – 21; pH: n = 7 – 26; membrane potential: n = 24 – 64.
Hatched columns: control values of verapamil-arrested hearts.

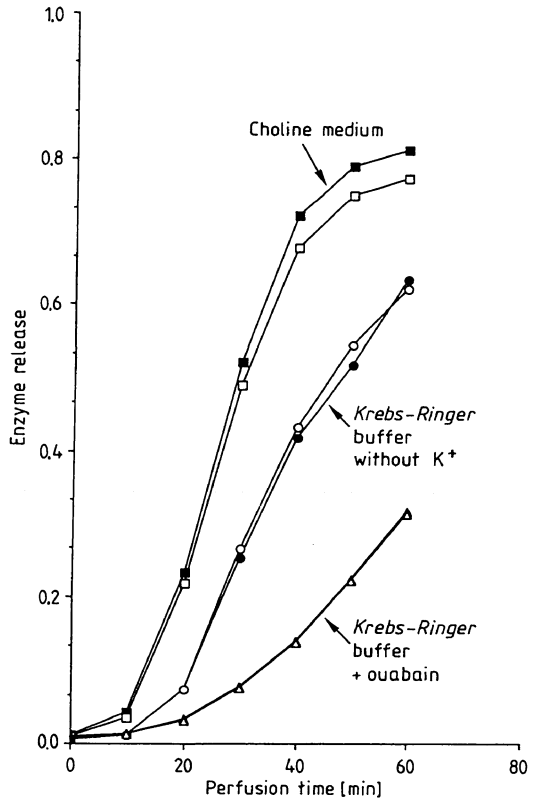

Fig. 7. Enzyme release (ratio of catalytic activity of perfusate over total catalytic activity of the myocardium) during normoxic perfusion at 0.1 mmol/l $[Ca^{2+}]_e$. Choline medium (contains 118 mmol/l choline chloride instead of 118 mmol/l sodium chloride): SEM $\leq \pm$ 0.07, n = 4; *Krebs-Ringer* medium without K^+: SEM $\leq \pm$ 0.052, n = 4; *Krebs-Ringer* medium + 10 µmol/l ouabain: SEM $\leq \pm$ 0.048, n = 4.
Closed symbols: lactate dehydrogenase;
open symbols: creatine kinase.

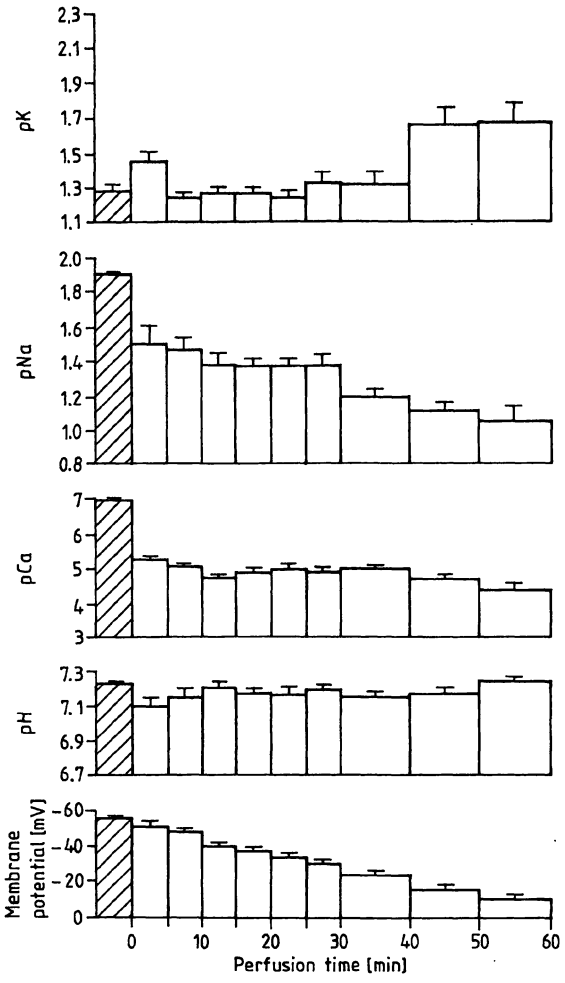

Fig. 8. Negative logarithms of sarcosolic cation concentrations and the membrane potential during normoxic perfusion at 0.1 mmol/l $[Ca^{2+}]_e$. *Krebs-Ringer* medium + 10 µmol/l ouabain. pK: n = 7 – 20; pNa: n = 7 – 18; pCa: n = 6 – 13; pH: n = 8 – 7; membrane potential: n = 31 – 62.
Hatched columns: control values of verapamil-arrested hearts.

Discussion

The membrane potential and $[K^+]_i$

Salt leakage from 3 mol/l KCl-filled microelectrodes having resistances of 16 MΩ may amount to 10 fmol/s (7). Measurements with ion-selective microelectrodes revealed that cytosolic KCl can increase at a rate of 70 mmol/l · min (8). The possibility cannot be excluded, therefore, that the more negative membrane potential found with microelectrodes containing 3 mol/l KCl was caused by salt leakage into the sarcosol. As a result $[K^+]_i$ increased and the associated membrane potential became hyperpolarised. The less negative membrane potential, which we found with high resistance micropipettes would be consistent with this view. In our opinion, the rather positive value of −57 mV found with 0.163 mol/l KCl-filled microelectrodes of low (double-barrelled) and high resistance may represent the more reliable result, although an increased interference by liquid junction and tip potentials has to be expected.

The much higher $[K^+]_i$ obtained by using 3 mol/l Li acetate as the reference electrolyte may be also explained by salt leakage. This would imply, however, that either Li^+ was exchanged against extracellular K^+, or an appreciable amount of K^+ was released from intracellular sites by the increasing sarcosolic Li acetate concentration.

Ischaemia and reperfusion, and the oxygen paradox

The kinetics of enzyme release during reperfusion and reoxygenation are nearly identical, which illustrates the similarity of both processes. In principle, this similarity exists also with respect to the change of sarcosolic cation concentrations. However, enzyme release under control conditions was more pronounced during reperfusion (fig. 1) than during reoxygenation (fig. 3). This difference may be explained by the fact that, during ischaemia, intracellular lactate accumulation is much greater than during hypoxic perfusion (9–12), whereby the osmolarity of myocardial cells under the former conditions may have been elevated above isotonicity. Additional cell swelling caused by water influx must have occurred immediately following reperfusion with isotonic medium. With respect to our hypothesis of cell damage (4), this swelling, occurring at a strongly elevated $[Ca^{2+}]_i$ (fig. 2), could initiate membrane blebbing and enzyme release under control conditions during reperfusion. This induction by swelling, however, must be of secondary importance during reperfusion with oxygen-containing *Krebs-Ringer* medium, because there was no significant difference between enzyme release under the latter conditions and during the reoxygenation phase of the oxygen paradox. Moreover, both processes proceeded at a much higher release rate. It is concluded, therefore, that myocardial cell swelling is not the trigger of cell damage at high $[Ca^{2+}]_i$ under the conditions of reperfusion after prolonged ischaemia, and reoxygenation after prolonged hypoxia.

Inhibition of ATP production under both conditions resulted in a decrease of enzyme release (figs. 1 and 3). This is in accordance with the results of *Ganote* et al. (13–15) and also with our earlier observations that ATP can markedly exaggerate enzyme release (4). During the reoxygenation phase of the oxygen paradox (fig. 3), evidently, the additional inhibition of glycolysis (oligomycin + deoxyglucose) caused a further decrease in enzyme release. If mitochondrial ATP

production was only partially inhibited by 2 μmol/l oligomycin, then a reduction of metabolic flux by deoxyglucose and thus a lowered proton flow through mitochondrial ATP-synthetase could also have produced a decrease in ATP production. However, we could not further decrease enzyme release by increasing the oligomycin concentration to 10 μmol/l. It is suggested, therefore, that in addition to mitochondrial ATP production, glycolytic ATP production is sufficient to trigger enzyme release at a high $[Ca^{2+}]_i$.

It is known from the thermodynamics of irreversible processes (16) that, for an ATP-coupled reaction, it is the power-output of ATP-hydrolysis, $A_{ATP} \cdot v$

($A_{ATP} = -dG/d\xi$ = affinity of ATP-hydrolysis;
$v = d\xi/dt$ = reaction velocity),

that causes the coupled reaction to be driven at a given velocity.

The total adenine nucleotide content of the myocardium may be very low after prolonged (> 60 min) ischaemia or hypoxia (17, 18), but the above facts indicate that A_{ATP} at very low ADP concentrations of the sarcosol may have been reelevated, so that $A_{ATP} \cdot v$ was sufficiently increased to induce cytoskeletal contractions at a markedly increased $[Ca^{2+}]_i$. This in turn, may have produced membrane blebbing and enzyme release as described in l. c. (4).

Antimycin A was most effective in reducing myocardial cell damage and enzyme release during ischaemia and reperfusion, as well as under the conditions of the oxygen paradox (figs. 1 and 3). In addition to inhibition of mitochondrial ATP production, the poison may indirectly have inhibited glycolysis. Inhibition of the mitochondrial respiratory chain, in addition to a lack of oxygen, may have led to a more complete and earlier collapse of the proton electrochemical potential difference ($\Delta\tilde{\mu}_H$) at the inner membrane. Reducing equivalents of the mitochondrial matrix, therefore, must have accumulated in the sarcosol via the $\Delta\tilde{\mu}_H$-dependent malate/aspartate-shuttle (19). The markedly decreased NADH redox potential of the sarcosol, in turn, may have inhibited glycolysis, so that lactate accumulation and associated swelling, especially during ischaemia, were almost completely prevented. In addition, an elevated NADH concentration is known to inhibit glyceraldehyde 3-phosphate dehydrogenase (20).

It is noteworthy in this context that under conditions of antimycin A-poisoning, cell damage with regard to enzyme release was lowest, although O_2-radical production by mitochondria under these same conditions is known to be activated (21, 22). This is also in accordance with the results of *Van der Heiden* et al. (23), who showed that enzyme release during the oxygen paradox was not reduced by scavengers of toxic oxygen metabolites, although cell injury caused by peroxidation was prevented.

Recovery from ischaemia by reperfusion is possible only about 30 min after the onset of injury (2). This time interval may be even shorter under hypoxic conditions. $[Ca^{2+}]_i$ was already markedly elevated during the intervals between 10 and 20 min (figs. 2 and 4). This is in accordance with the onset of an increasing resting tension under similar conditions reported by *Dixon* et al. (24). In contrast, using radioactive tracer methods, *Poole-Wilson* et al. (25) could not demonstrate such an early increase of the myocardial Ca^{2+} content. It seems reasonable to suggest, therefore, that the early increase of $[Ca^{2+}]_i$, which we observed, was caused by a release of Ca^{2+} from intracellular stores, most probably from the sarcoplasmatic reticulum. *Kammermeier* has suggested (26) that early hypoxic failure may be brought about by a decrease of A_{ATP} and an associated reduction primarily of Ca^{2+} pumping of the sarcoplasmatic reticulum ATPase. Because this transport reaction is near equilibrium under resting conditions (27), even a small decrease of A_{ATP} may be followed by a reduction of $\Delta\mu_{Ca}$ at the reticulum membrane, and therefore also by an increase of $[Ca^{2+}]_i$.

With respect to our hypothesis, recovery should be possible if $[Ca^{2+}]_i$ could be lowered to a tolerable value before $A_{ATP} \cdot v$ of the sarcosol is reelevated, provided that ADP is available (under ischaemic conditions additional cell swelling must be prevented during reperfusion). Ca^{2+} is transported by the mitochondrial uniporter ($K_m \sim 10$ μmol/l) with preference over ATP synthesis, whereby $[Ca^{2+}]_i$ may have been decreased sufficiently before $A_{ATP} \cdot v$ was increased again. The results of figures 2 and 4 show, however, that $[Na^+]_i$ was also markedly elevated so that Ca^{2+} entry via reactivated Na/Ca exchange at the sarcolemma must be considered (18, 28–30), especially when $[Ca^{2+}]_i$ may be lowered by mitochondria at the onset of reperfusion or reoxygenation.

It is uncertain whether Na/Ca exchange transport at the mitochondrial inner membrane is substantially involved with the adjustment of an elevated set point of $[Ca^{2+}]_i$, because this exchange process may be inhibited at an elevated $[Ca^{2+}]_i$ (31, 32).

Inward transport of Ca^{2+} via Na/Ca exchange at the sarcolemma is possible when the driving force of this

reaction becomes negative. Under these conditions, mitochondrial Ca^{2+} transport may be, at least in part, compensated by Ca^{2+} influx, so that $[Ca^{2+}]_i$ cannot be decreased efficiently. During hypoxia, this situation was reached after about 40 min (tab. 1). If, however, a value of pCa = 4.7 (= 20 µmol/l) is taken as an upper limit for a tolerable $[Ca^{2+}]_i$ (see below), Ca^{2+} influx must be considered as occurring as early as 30 min after the initiation of hypoxic conditions. Prior to 30 min the driving force is positive, so that Ca^{2+} cannot enter the myocyte and recovery by reoxygenation is therefore possible.

Beyond this time interval and up to about 60 min of hypoxia, reoxygenation did not initiate a drastic enzyme release (preliminary results not shown). Supposedly, $[Ca^{2+}]_i$ was maintained below the critical concentration by mitochondria, a complete recovery, however, was impossible, because ATP production may have been insufficient to lower $[Na^+]_i$ and to reverse the Na/Ca reaction.

The sudden cell damage occurring after prolonged ischaemia or hypoxia (figs. 1 and 3) may then be explained by the fact that, on the one hand, at a very low sarcosolic ADP and lowered phosphate concentration (by mitochondrial phosphate accumulation), a sufficiently high $A_{ATP} \cdot v$ can be attained very rapidly, and on the other hand, at the highly increased $[Ca^{2+}]_i$ and the markedly negative driving force of the Na/Ca exchange reaction, the mitochondrial Ca^{2+} accumulation rate was no longer sufficient to decrease $[Ca^{2+}]_i$ to a tolerable value. According to l. c. (4), enzyme release under these conditions is induced by ATP at a high $[Ca^{2+}]_i$.

Reduced extracellular calcium

Electron- and light-microscopic observations by *Muir* (33) have shown that myocytes of rat heart tissue became dissociated when perfused with media containing decreased concentrations of Ca^{2+} ($\leq$ 0.25 mmol/l). We have found that a $[Ca^{2+}]_e < 0.3$ mmol/l induces swelling and enzyme release in choline media. It is reasonable to suggest, therefore, that the $[Ca^{2+}]_e$-dependent dissociation of myocytes creates additional leaks for small ions and molecules. This interpretation is also consistent with the results of *Haas* et al. from conductivity measurements (34). A rapid accumulation of ions and water through these leaks can then occur through cellular *Donnan* forces.

Under hypoxic conditions, the *Donnan* swelling at a markedly elevated $[Ca^{2+}]_i$ may have induced cell damage and enzyme release. Comparing figures 5 and 6, it is evident that the drastic increase of enzyme release (*Krebs-Ringer* medium) was induced after 20 min of perfusion and that a further marked elevation of $[Ca^{2+}]_i$ was likewise found after this time. It is suggested, therefore, that the critical $[Ca^{2+}]_i$ which is necessary to initiate the blebbing process through swelling or ATP (4) may fall in the range between pCa = 5 and pCa = 4.5 (10 and 32 µmol/l) (fig. 6). The acceleration of enzyme release by verapamil is more difficult to explain (fig. 5). However, it is consistent with the above idea of additional leaks, since blocking of Ca^{2+} channels by this agent was not protective.

The earlier onset of enzyme release by perfusion with choline media (fig. 5) was supposedly brought about by a more rapidly increasing $[Ca^{2+}]_i$. This assumption is supported by the observation that Na/Ca exchange during the first few minutes compensated for the Ca^{2+} influx when $[Na^+]_e$ was normal, but less efficiently when $[Na^+]_e$ was low. This may also explain the most pronounced enzyme release during normoxic conditions (fig. 7). The much higher release rate under the conditions of normoxic choline perfusion, as compared to the analogous hypoxic conditions (figs. 5 and 7), may have been caused by an ATP-induced membrane blebbing at a high $[Ca^{2+}]_i$ which may have accelerated enzyme release (4).

Wittenberg has shown (35) that when hearts were perfused with oxygen- and glucose-containing *Krebs-Ringer* medium containing 0.1 mmol/l Ca^{2+} for 30 min, sarcosolic pNa and pH values were only slightly decreased (1.824 and 7.145, respectively), and $[Ca^{2+}]_i$ remained fairly constant at about 0.9 µmol/l. The present results demonstrate that inhibition of the Na/K pump by ouabain resulted in a pronounced change, especially of pNa and pCa under analogous conditions (fig. 8). Therefore, it is suggested that it is primarily the Na/Ca exchange reaction at a functioning Na/K pump which is responsible for Ca^{2+} outward transport and, thus, for maintenance of cell integrity under conditions of a leaky cell membrane.

Acknowledgement

The expert technical assistence of *M. Fischer* and *R. Nilson-Neugebauer* is gratefully acknowledged.

References

1. Alto, L. E. & Dhalla, N. S. (1979) Myocardial cation contents during induction of calcium paradox. Am. J. Physiol. *237*, H713–H719.
2. Reimer, K. A. & Jennings, R. B. (1986) Myocardial ischemia, hypoxia, and infarction. In: *The Heart and Cardiovascular System* (Fozzard, H. A., ed.) pp. 1133–1201, Raven Press, New York.
3. Grinwald, P. M. & Brosnahan, C. (1987) Sodium imbalance as a cause of calcium overload in post-hypoxic reoxygenation injury. J. Mol. Cell. Cardiol. *19*, 487–495.
4. Diederichs, F., Mühlhaus, K., Wittenberg, H. & Trautschold, I. (1986) Enzyme release from the perfused rat heart. The functions of the cytoskeleton under cell-pathological conditions. J. Clin. Chem. Clin. Biochem. *24*, 3–9.
5. Diederichs, F. & Wittenberg, H. (1986) Intracellular free Ca^{2+} in the course of the Ca^{2+} paradox and during poisoning. Ca^{2+}-selective microelectrode measurements in the perfused rat heart. J. Clin. Chem. Clin. Biochem. *24*, 971–979.
6. Ammann, D., Morf, W. E., Anket, P., Meier, P. C., Pretsch, E. & Simon, W. (1983) Neutral carrier based ion-selective electrodes. Ion-Selective Electrode Rev. *5*, 3–92.
7. Fromm, M. & Schultz, S. G. (1981) Some properties of KCl-filled microelectrodes: Correlation of potassium "leakage" with tip resistance. J. Membrane Biol. *62*, 239–244.
8. Blatt, M. R. & Slayman, C. L. (1983) KCl leakage from microelectrodes and its impact on the membrane parameters of a nonexcitable cell. J. Membrane Biol. *72*, 223–234.
9. Tranum-Jensen, J., Janse, M. J., Fiolet, J. W. T., Krieger, W. J. G., D'Alnoncourt, N. C. & Durrer, D. (1981) Tissue osmolality, cell swelling, and reperfusion in acute regional myocardial ischemia in the isolated porcine heart. Circulation Res. *49*, 364–381.
10. Jennings, R. B., Schaper, J., Hill, M. L., Steenbergen, C. & Reimer, K. A. (1985) Effect of reperfusion late in the phase of reversible ischemic injury Changes in cell volume, electrolytes, metabolites, and ultrastructure. Circulation Res. *56*, 262–278.
11. Steenbergen, C., Hill, M. L. & Jennings, R. B. (1985) Volume regulation and plasma membrane injury in aerobic, anaerobic, and ischemic myocardium in vitro. Effects of osmotic cell swelling on plasma membrane integrity. Circulation Res. *57*, 864–875.
12. Jennings, R. B., Reimer, K. A. & Steenbergen, C. (1986) Myocardial ischemia revisited. The osmolar load, membrane damage, and reperfusion. J. Mol. Cell. Cardiol. *18*, 769–780.
13. Ganote, C. E., Worstell, J. & Kaltenbach, J. P. (1976) Myocardial ischemia revisited. The osmolar load, membrane damage, and reperfusion. Am. J. Pathol. *84*, 327–350.
14. Ganote, C. E., McGarr, J., Liu, S. Y. & Kaltenbach, J. P. (1980) Oxygen-induced enzyme release. Assessment of mitochondrial function in anoxic myocardial injury and effects of the mitochondrial uncoupling agent 2,4-dinitrophenol (DNP). J. Mol. Cell. Cardiol. *12*, 387–408.
15. Ganote, C. E., Editorial (1983) Contraction band necrosis and irreversible myocardial injury. J. Mol. Cell. Cardiol. *15*, 67–73.
16. Caplan, S. R. & Essig, A. (1983) *Bioenergetics and linear nonequilibrium thermodynamics: The steady state*. Harvard University Press, Cambridge, Massachusetts, and London.
17. Jennings, R. B. & Steenbergen, C. (1985) Nucleotide metabolism and cellular damage in myocardial ischemia. Ann. Rev. Physiol. *47*, 727–749.
18. Allen, D. G. & Orchard, C. H. (1987) Myocardial contractile function during ischemia and hypoxia. Circulation Res. *60*, 153–168.
19. Nicholls, D. G. (1982) *Bioenergetics: An Introduction to the Chemiosmotic Theory*. Academic Press, London, New York.
20. Mochizuki, S. & Neely, J. R. (1979) Control of glyceraldehyde-3-phosphate dehydrogenase in cardiac muscle. J. Mol. Cell. Cardiol. *11*, 221–236.
21. Chance, B., Sies, H. & Boveris, A. (1979) Hydroperoxide metabolism in mammalian organs. Physiological Rev. *59*, 527–603.
22. Nohl, H. & Jordan, W. (1986) The mitochondrial site of superoxide formation. Biochem. Biophys. Res. Commun. *138*, 533–539.
23. Vander Heiden, R. S., Sobotka, P. A. & Ganote, C. E. (1987) Effects of the free radical scavenger DMTU and mannitol on the oxygen paradox in perfused rat hearts. J. Mol. Cell. Cardiol. *19*, 615–625.
24. Dixon, I. M. C., Eyolfson, D. A. & Dhalla, N. S. (1987) Sarcolemmal Na^+-Ca^{2+} exchange activity in hearts subjected to hypoxia reoxygenation. Am. J. Physiol. *253*, H1026–H1034.
25. Poole-Wilson, P. A., Harding, D. P., Bourdillon, P. D. V. & Tones, M. A. (1984) Calcium out of control. J. Mol. Cell. Cardiol. *16*, 175–187.
26. Kammermeier, H. (1987) High energy phosphate of the myocardium: Concentration versus free energy change. Bas. Res. Cardiol. *82*, Suppl. 2, 31–36.
27. Hasselbach, W. & Oetliker, H. (1983) Energetics and electrogenicity of the sarcoplasmatic reticulum calcium pump. Ann. Rev. Physiol. *45*, 325–339.
28. Caroni, P. & Carafoli, E. (1983) The regulation of the Na^+-Ca^{2+} exchanger of heart sarcolemma. Eur. J. Biochem. *132*, 451–460.
29. Carafoli, E. (1985) The homeostasis of calcium in heart cells. J. Mol. Cell. Cardiol. *17*, 203–212.
30. McGuigan, J. A. S. & Blatter, L. A. (1987) Sodium/calcium exchange in ventricular muscle. Experientia *43*, 1140–1145.
31. Hayat, L. H. & Crompton, M. (1982) Evidence for the existence of regulatory sites for Ca^{2+} on the Na^+/Ca^{2+} carrier of cardiac mitochondria. Biochem. J. *202*, 509–518.
32. Hayat, L. H. & Crompton, M. (1985) Ca^{2+}-dependent inhibition by trifluoperazine of the Na^+-Ca^{2+} carrier in mitoplasts derived from heart michochondria. FEBS Lett. *182*, 281–286.
33. Muir, A. R. (1967) The effects of divalent cations on the ultrastructure of the perfused rat heart. J. Anat. *101*, 239–261.
34. Haas, H. G., Meyer, R., Einwächter, H. M. & Stockem, W. (1983) Intercellular coupling in frog heart muscle. Electrophysiological and morphological aspects. Pflügers Arch. *399*, 321–335.
35. Wittenberg, H. (1985) Cytosolische Kationen-Homöostase in der Herzmuskelzelle. Intrazelluläre Messungen der Na^+-H^+- und Ca^{2+}-Aktivität mit ionenselektiven Mikroelektroden am isoliert perfundierten Rattenherzen. Thesis Medical School Hannover.

Dr. rer. nat. Frank Diederichs
Abteilung für Klinische Biochemie
Medizinische Hochschule Hannover
Konstanty-Gutschow-Str. 8
D-3000 Hannover 61